Seedless Watermelons Produced Via Soft-X-Irradiated Pollen

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Abstract. Watermelon fruit that results from pollination with pollen irradiated with soft-X-ray contains only empty seed, although the fruit develops to a normal size. In this study the processes of fertilization and embryo formation were compared between unirradiated and irradiated pollen in order to reveal the mechanisms by which seedless fruits are generated. The use of soft-X-irradiated pollen resulted in normal pollen tube penetration into the synergid and discharge of sperm cells. Two to three days after pollination, the sperm nucleus was present alongside the egg nucleus before fusion. The polar nuclei divided and the endosperm cell spread in the embryo sac before zygote division. A globular embryo was observed on day 7 to 10 after pollination. Subsequently, the embryo failed to differentiate to organ tissue and degenerated. These results indicate that double fertilization occurred after pollination with the irradiated pollen and that abortion of the embryo results from soft-X-ray induced chromosomal abnormalities in generative nucleus.

We have developed a new method for producing seedless watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai] in diploid plants using soft-X-irradiated pollen (Sugiyama and Morishita, 1998).

Watermelon fruits that result from pollination with pollen irradiated with 800 Gy or more of soft-X-rays contain only empty seeds and are devoid of normal seeds (Sugiyama and Morishita, 2000a). Generally, irradiated pollen germination rate is greatly reduced (Sari et al., 1992) and fruit set rate following pollination with irradiated pollen is reduced compared to that following pollination with unirradiated pollen (Sharma and Haq, 1989). In tomato (Umatsen and Nishiyama, 1967) and French bean (Sharma and Haq, 1989), parthenocarpic fruits produced by using irradiated pollen are very small. However, the rate of pollen germination and fruit set are hardly affected by soft-X-irradiation in our set-up condition (Sugiyama and Morishita, 2000a, 2000b). In addition, diploid seedless fruit is similar in size to control fruit (Sugiyama and Morishita, 2000a, 2000b).

Previous studies have been reported on the induction of parthenogenesis in haploid or diploid embryos (Pandey and Phung, 1982; Pandey, 1990; Sauton and Dumas de Vaulx, 1987;

Truong-Andre, 1988; Sari et al., 1994) and on gene transformation (Pandey, 1975, 1978) through the use of irradiated pollen. However, few anatomical studies of the embryo sacs after fertilization with irradiated pollen have been performed (Stadler, 1928; Steinitz-Sears and Sears, 1957; Vassileva-Dryanovska, 1966a, 1966b) and none of these studies was carried out on cucurbit crops. In this study, the processes of fertilization and embryonic development in the pollinated ovule were analyzed in the watermelon following pollination with soft-X-irradiated pollen, in order to elucidate the parthenocarpic mechanisms involved.

Materials and Methods

The main watermelon cultivar 'Fujihikari TR' in Japan was sown on 3 Mar., 1998. The male flowers were picked on the morning of anthesis. The blooming male flowers were irradiated with a 800 Gy dose (11.1 Gy/min) of soft-X-ray (Unit OM-60R; OHMIC, Tokyo) and were immediately used for pollination. An 800 Gy dose was selected on the basis of previous findings (Sugiyama and Morishita, 2000a). Unirradiated pollen was used as a control.

A minimum of five fruits was selected at random at 0, 24, 30, 36, 42, 48, 54, and 60 h and 3, 4, 5, 7, 8, 10, 12, 14, 16, 18, and 20 d after pollination with soft-X-irradiated pollen or unirradiated pollen for observation of fertilization and embryo formation.

For 5 d after pollination (0, 24, 30, 36, 42, 48, 54, and 60 h and 3, 4, and 5 d), five watermelon ovaries were cut equatorially and rapidly fixed with FAA solution (formalin-acetic acid-alcohol). About 250 ovaries were

observed in all the treatments. Next, the five largest ovules were dissected from five ovaries on days 7, 8, 10, 12, 14, 16, 18, and 20 after pollination and fixed in FAA solution. Subsequently, the fixed specimens were dehydrated in a graded t-butanol series, embedded in paraffin, sliced into 10-mm-thick serial sections with a rotating microtome and mounted onto glass slides. The mounted sections were stained with haematoxylin and safranin, or with haematoxylin, safranin, and fast green. Subsequently, the stained sections were dehydrated in a graded ethanol series and the ethanol was replaced with xylene using a serial dilution. The slide were coverslipped using balsam prior to microscopy.

Results

Process of fertilization. The embryo sac of watermelon is illustrated in Fig. 1A. It contains two synergids, an egg cell, a central cell with two polar nuclei, and three antipodal cells.

Using unirradiated pollen, pollen tube penetration into one of the two synergids at 30 to 36 h after pollination (Fig. 1B) and discharge of sperm nuclei occurred normally. At 1 to 3 d after flowering, a sperm nucleus was seen inside the egg cell before fusion (Fig. 1C). The other sperm nucleus in the same embryo sac was seen adjacent to the two polar nuclei.

Using irradiated pollen, at 30 to 36 h after pollination, pollen tube penetration into one of the two synergids and discharge of sperm nuclei appeared to occur normally (Fig. 1D), and by 2 to 3 d after pollination, a sperm nucleus was seen alongside the egg nucleus before fusion (Fig. 1E).

Development of embryo. Following pollination with the unirradiated pollen, the zygote divided as early as 3 to 4 d after pollination. The proembryo and globular embryo formed 5 to 7 d after pollination (Fig. 2A and B), and a heart-shaped embryo was observed 8 d after pollination (Fig. 2C). By days 12 to 16, the embryo had formed two cotyledons, radicle, epicotyl, and hypocotyl, and subsequently developed into a mature embryo (Fig. 2D).

In contrast, there was a slight delay in the development of the proembryo following pollination with irradiated pollen in comparison with that following pollination with unirradiated pollen. After pollination with irradiated pollen, the zygote divided on days 4 to 5, and the proembryo and globular embryo were observed on days 7 to 10 (Fig. 3A and B). There was also a reduction in the number of cells in the globular embryo following pollination with irradiated pollen, and the shape of the embryo was altered (Fig. 3C). A typical heart-shaped embryo was not observed. At about day 10, the embryo degenerated and did not form the typical embryonic organs (Fig. 3D).

Development of the endosperm. One of the sperm nuclei could be seen alongside the polar nuclei in both the unirradiated and irradiated pollen on day 2 after pollination. The

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Fig. 1. Process of double fertilization with unirradiated and irradiated pollen. (A) The embryo sac of a large ovule at flowering (day 0). (B) Section of embryo sac on day 2 (30 h) after pollination with unirradiated pollen. A pollen tube penetrating into a synergid cell and discharge of the sperm nucleus, can be seen. (C) Section of embryo sac on day 3 (72 h) after pollination with unirradiated pollen, showing an egg nucleus and sperm nucleus attached in the egg cell and endosperm nuclei. Endosperm nuclei have expanded in the embryo sac. (D) Section of embryo sac on day 2 (42 h) after pollination with irradiated pollen, showing a pollen tube penetrating into a synergid cell, and discharge of sperm nucleus. (E) Section of embryo sac on day 3 (72 h) after pollination with irradiated pollen, showing an egg nucleus and sperm nucleus (triangular arrows) attached in the egg cell. Endosperm nuclei have spread in the embryo sac. Horizontal bar: (A)=10 μ m, (B-E)=25 μ m. Abbreviations: ac = antipodal cell, ec = egg cell, en = egg nucleus, esn = endosperm nuclei, pn = polar nucleus, pt = pollen tube, sc = synergid cell, sn = sperm nucleus.

nucleus of the central cell, which would fuse with the sperm nucleus, divided by free nuclear divisions. There were 4 to 8 endosperm nuclei 42 h after pollination. After this time the dividing endosperm nuclei extended along the outer line of the embryo sac (Fig. 1 C and E).

Following pollination with either unirradiated or irradiated-pollen, the globular embryo was surrounded by endosperm cells at days 5 to 7 (Fig. 2B, Fig. 3B). In the fruit that resulted from pollination with unirradiated

pollen, the endosperm cells gradually disappeared with the growth of the embryo starting on days 8 to 10 (Fig. 2C). However, in the fruit that resulted from pollination with irradiated pollen, the endosperm cells remained until day 16 in most of the ovules.

Discussion

The division of generative nuclei in *Lilium regale* pollen tubes was reported to be inhibited by exposure to 220 Gy (Price, 1957). In

Tradescantia, various abnormalities were observed in pollen tube mitoses. The generative nucleus did not often divide after exposure of *Tradescantia* pollen to 100 to 500 Gy (Vassileva-Dryanovska, 1966a), and exposure of *Lilium* pollen to 50 to 500 Gy (Vassileva-Dryanovska, 1966b). In addition, after the pollen tube reached the interior of the embryo sac, single fertilization occurred, because the generative nucleus did not divide after exposure of pollen to 500 to 5000 Gy in both species. Vassileva-Dryanovska reported

Fig. 2. Process of embryo growth following pollination with unirradiated pollen. (A) Section of embryo sac on day 4, showing proembryo formation and endosperm nuclei. (B) Section of embryo sac on day 5, showing globular embryo. (C) Section of embryo sac on day 8, showing heart-shaped embryo. (D) Section of embryo sac on day 16, showing that the embryo has formed cotyledons, radicle, hypocotyl, and shoot hypocotyl. Horizontal bar: (A) = 50 μ m, (B) 25 μ m, (C, D) = 250 μ m. Abbreviations: am = apical meristem, c = cotyledons, es = endosperm, esn = endosperm nuclei, ge = globular embryo, h = hypocotyl, pe = proembryo, r = radicle.

that haploid embryos may develop after stimulation of the egg nucleus to divide by the pycnotic male chromatin, or may be produced without fertilization under the influence of the developing endosperm after irradiation in both *Tradescantia* (Vassileva-Dryanovska, 1966a) and *Lilium* (Vassileva-Dryanovska, 1966b). Subsequently, embryo and endosperm were found to degenerate in the later stages.

In a previous study of watermelon, discharge of sperm nuclei occurred 2 d after pollination with normal pollen (Buttrose and

Sedgley, 1979). In our study, a similar finding was observed 36 to 42 h after pollination with irradiated pollen. Embryonic development in watermelon was reported to occur in pollinated, but not in unpollinated (Buttrose and Sedgley, 1979) or auxin-induced parthenocarpic ovules (Sedgley et al., 1977). We presumed, therefore, that fertilization following sperm cell discharge occurs equally with soft-X-irradiated pollen as with unirradiated-pollen.

Sari et al. (1994) reported that haploid embryos were obtained after in vitro culture of proembryos following pollination with

300 Gy γ -ray-irradiated pollen in watermelon. It is well known that γ - or X-irradiation of pollen may result in induced genetic abnormalities such as gene mutation, chromosomal rearrangement and breakage of chromosome in generative nuclei. Regarding the abortion of embryos after fertilization by soft-X-irradiated pollen, we postulate that the abortion of the embryo results from chromosomal abnormalities induced with soft-X-ray in generative nucleus.

The developmental formation of the endosperm was normal in the early stages following pollination with soft-X-irradiated pollen. Buttrose and Sedgley (1979) reported that in nonpollinated ovaries and in auxin-induced parthenocarpic ovaries, the polar nuclei were degenerated and did not divide. Furthermore, in watermelon, endosperm development occurred in pollinated, but not in unpollinated or auxin-induced parthenocarpic ovules (Sedgley et al., 1977). Our results indicate that fertilization had occurred in the central cell after pollination with soft-X-irradiated pollen.

Parthenocarpic watermelon fruits induced by hormonal agents have a tendency to be small and deformed compared to normal fruit (Hayata et al., 1995; Kondou and Murozono, 1975). In contrast, the seedless watermelon fruit produced by soft-X-irradiated pollen were normal in size and shape. Ovary development is promoted by gibberellin and cytokinin secreted by the numerous developing embryos after fertilization (Okamoto, 1996). It is probable, therefore, that diploid seedless watermelon fruit developing after pollination with soft-X-irradiated pollen develop to full size because parthenocarp is induced by the growth hormones secreted by pollen grains and the ovules.

We have followed the process of empty seed formation in the ovary after pollination with soft-X-irradiated pollen. In addition, we have shown that double fertilization must occur after pollination with soft-X-irradiated pollen, and that embryonic development continues up to the stage of the globular embryo. However subsequent embryonic growth does not occur and the embryo degenerates. As ovules with degenerative embryos do not grow, they fail to reach their normal size and do not form a hard seed coat. It would be informative to investigate whether all or part of the chromosomes in sperm nuclei are involved in nuclear fusion. Other outstanding questions include the behavior of the chromosomes of the sperm nucleus during embryonic development and the effects of growth hormones secreted from degenerating seeds in the process of fruit development.

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Fig. 3. Process of embryo growth following pollination with irradiated pollen. (A) Section of embryo sac on day 5, showing proembryo formation and endosperm nuclei. (B) Section of embryo sac on day 7, showing globular embryo. (C) Section of embryo sac on day 12 after pollination, showing a degenerate globular embryo. (D) Section of embryo sac on day 20, showing an aborted embryo. Horizontal bar: (A, D) = 50 μ m, (B, C) = 25 μ m. Abbreviations: ae = aborted embryo, dge = degenerate globular embryo, esn = endosperm nuclei, ge = globular embryo, pe = proembryo.

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